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Polar Compounds Dominate in Vitro Effects of Sediment Extracts

Urte Lübcke-von Varel,[†] Miroslav Machala,[‡] Miroslav Ciganek,[‡] Jiri Neca,[‡] Katerina Pencikova,[‡] Lenka Palkova,[‡] Jan Vondracek,[‡] Ivonne Löffler,[†] Georg Streck,[†] Georg Reifferscheid,[§] Sini Flückiger-Isler,[⊥] Jana M. Weiss,[#,¶] Marja Lamoree,[¶] and Werner Brack[†,*]

[†]Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany,

[‡]Veterinary Research Institute, Brno, Czech Republic,

[§]Federal Institute of Hydrology, Koblenz, Germany,

[⊥]Xenometrix AG, Allschwil, Switzerland,

[¶]Institute for Environmental Studies, VU University Amsterdam, The Netherlands

Supporting Information

ABSTRACT: Sediment extracts from three polluted sites of the river Elbe basin were fractionated using a novel online fractionation procedure. Resulting fractions were screened for mutagenic, aryl hydrocarbon receptor (AhR)-mediated, transthyretin (TTR)-binding, and estrogenic activities and their potency to inhibit gap junctional intercellular communication (GJIC) to compare toxicity patterns and identify priority fractions. Additionally, more than 200 compounds and compound classes were identified using GC-MS/MS, LC-MS/MS, and HPLC-DAD methods. For all investigated end points, major activities were found in polar fractions, which are defined here as fractions containing dominantly compounds with at least one polar functional group. Nonpolar PAH fractions contributed to mutagenic and AhR-mediated activities while inhibition of GJIC and estrogenic and TTR-binding activities were exclusively observed in the polar fractions. Known mutagens in polar fractions included nitro- and dinitro-PAHs, azaarenes, and keto-PAHs, while parent and monomethylated PAHs such as benzo[*a*]pyrene and benzofluoranthenes were identified in nonpolar fractions. Additionally, for one sample, high AhR-mediated activities were determined in one fraction characterized by PCDD/Fs, PCBs, and PCNs. Estrone, 17β-estradiol, 9*H*-benz[*de*]anthracen-7-one, and 4-nonylphenol were identified as possible estrogenic and TTR-binding compounds. Thus, not only nonpolar compounds such as PAHs, PCBs, and PCDD/Fs but also the less characterized and investigated more polar substances should be considered as potent mutagenic, estrogenic, AhR-inducing, TTR-binding, and GJIC-inhibiting components for future studies.

INTRODUCTION

Contaminated sediments may exhibit a number of hazardous effects. Some of them are in the focus of environmental research already for decades such as mutagenicity and aryl hydrocarbon receptor (AhR)-mediated effects, which may result in developmental and reproductive toxicity and carcinogenicity. Others were considered only more recently in the assessment of individual toxicants and environmental samples such as estrogenicity,[1] changes in thyroid hormone metabolism by competition with thyroxine for transthyretin (TTR) binding,[2] and the inhibition of gap-junctional intercellular communication (GJIC) that is believed to be an important step in tumor promotion and thus carcinogenesis.[3,4]

Classical target analysis often fails to identify the compounds causing these effects because of the enormous number of compounds accumulated in sediments and a lack of knowledge on their toxicological properties.[5] Combining high-throughput in vitro bioassays with fractionation techniques helps to associate effects to groups of contaminants with similar physicochemical properties and thus to prioritize individual fractions for subsequent effect-directed analysis (EDA).

Recently, based on a previous off-line normal-phase (NP) high performance liquid chromatography (HPLC) approach for sequential multistep fractionation of sediment extracts,[6] an improved and online fractionation procedure was developed that allows the class separation of major sediment-associated toxicants in one run combining three automatically switched normal-phase columns including cyanopropyl (CN), nitrophenyl (NO), and porous graphitized carbon (PGC).[7] The system was designed to provide 18 fractions coeluting with major halogenated aromatic compound classes such as PCBs, PCNs, and PCDD/Fs with increasing planarity and degree of chlorination, PAHs with increasing numbers of aromatic carbon atoms, and several more polar compound groups including nitro-PAHs, azaarenes, and PAH-quinones. Nonpolar compounds as defined in this study include hydrocarbons and halogenated hydrocarbons. Compounds with polar functional groups (e.g., keto, hydroxy, thiol, nitro, and amino groups) are defined as polar compounds. For the first time, combining an automated fractionation procedure with a battery of in vitro bioassays allowed the screening of sediment extracts from several sites for fraction-specific adverse effects, applying a broad range of toxicological end points.

The present study aimed (1) to test this novel effect-directed fractionation procedure for its suitability to characterize

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sediment contaminations, (2) to identify and compare endpoint-specific toxicity patterns from different contaminated sites, (3) to identify priority fractions for future research, and (4) to draw first conclusions on compound groups that may be responsible for the observed effects. The study was based on a battery of bioassays covering five toxicological end points. The applied bioassays include testing for mutagenicity using the Ames fluctuation assay[8,9] with tester strains TA98 and TA100 with and without metabolic activation, AhR-mediated activity applying the DR-CALUX assay,[10] determination of estrogenic activity by ER-CALUX,[11] thyroid hormone transport disturbing potency applying the transthyretin (TTR)-binding assay based on human TTR,[2] and inhibition of GJIC using rat epithelial WB-F344 cells.[3] Sediments from three sampling sites at industrial hot spots in the Elbe river basin were selected including the Elbe river in (1) Přelouč (P) downstream of the industrial area of Pardubice, Czech Republic, (2) the tributary Bílina downstream of the industrial area of Litvínov and Most (M), Czech Republic, and (3) the creek Spittelwasser downstream of Bitterfeld (B), Germany, one of the most important industrial areas of the German part of the Elbe basin.

EXPERIMENTAL SECTION

Sample Collection and Preparation. Sediment samples were collected in 2005 and 2006 using an Ekman-Birge-grab. Sampling sites were chosen according to known sources of contamination, to the availability of sediment, and to their accessibility. For additional information on sampling sites see also the Supporting Information.

All sediments were freeze-dried, sieved ($\leq$63 μm), and homogenized. Sediments were extracted using accelerated solvent extraction (ASE 300, Dionex Corp., Sunnyvale, CA) with dichloromethane:acetone 3:1 and dialyzed through a semipermeable membrane as described in detail elsewhere.[7] Residual water was removed by Na_2SO_4. Sediments intended for the TTR-binding assay were extracted using a slightly modified two-step extraction procedure: (1) hexane:dichloromethane (HX:DCM) 1:1 (v:v), 80 °C, (2) toluene, 140 °C, each with 3 cycles at 10 min static extraction time and 103 bar. After exchanging the solvent to DCM, extracts were purified with gel-permeation chromatography (Biobeads SX3, Bio Rad, Munich, Germany). All extracts were evaporated to dryness and redissolved in HX:DCM 9:1.

Fractionation. Compounds were separated according to their physicochemical properties using an automated multistep fractionation method based on three connected and automatically switched normal phase HPLC columns. The HPLC fractionation system has been described in detail elsewhere.[7] Polar polycyclic aromatic hydrocarbons (PACs) are trapped on and eluted from CN, while nonpolar PACs are retained on and eluted from NO and PGC stationary phases. Extracts were fractionated in aliquots of 40 g sediment equivalents (SEQ) per run. Corresponding fractions of different runs were pooled, reduced to dryness, redissolved in DCM, and divided into several aliquots for biotesting and chemical analysis. Aliquots were solvent-exchanged to the respective solvent.

Chemical and Biological Analysis. Fractions, nonfractionated parent and reconstituted extracts, were tested for mutagenic effects, AhR-mediated, estrogenic, and TTR binding activities, and the inhibition of GJIC. Reconstituted extracts were prepared, combining equal amounts of each fraction of a sample. Samples were screened for a broad range of compounds using GC-MS, LC-MS/MS, and HPLC DAD methods. For details on bioassays and analytical procedures, see the Supporting Information.

RESULTS AND DISCUSSION

Chemical Screening Analysis. Fractions were screened using LC-MS/MS, HPLC/DAD, and GC-MS instruments for target analytes and unknowns (Table S1, Supporting Information). The sediment samples exhibited different contamination profiles. The Přelouč sample is characterized by high concentrations of small petrogenic alkylated PAHs and sulfur heterocyclic derivatives, indicating a contamination with oil. Other outstanding contaminants were ethylpyridylindole, diphenylpropenethiols, and chloro- and dichloroanthraquinones as well as 1,3-dinitropyrene. The sediment from Most shows a greater contamination with pyrogenic five- and six-ring PAHs, while the Bitterfeld sample is characterized by high amounts of DDT derivatives and HCH isomers together with some other specific compounds that have been produced in Bitterfeld such as *N*-phenyl-2-naphthylamine, prometryn, methoxychlor, and methylparathion.

The chemical screening analysis was in agreement with the fractionation scheme based on standard compounds.[7] Compounds with small aromatic structures such as alkyl benzenes, naphthalenes, biphenyls, diphenyl ethers, dibenzofurans, PCNs, and PCBs occurred in fractions F2 to F5. Fractions 6 to 13 contained PAHs, alkyl-PAHs, and their oxygen (O)- and sulfur (S)-containing heterocyclic derivatives with an increasing number of aromatic carbons. Compounds with increasing polarity such as nitro-, keto-, and hydroxy-PAHs and nitrogen-containing heterocyclic aromatic compounds (N-PACs) were eluted in F13 to F18.

While DDT and metabolites eluted exclusively in the nonpolar F3 and F6 to F9, different HCH-isomers were found in nonpolar F7 and F8 as well as in the more polar F13 and F14 due to different polarities of the molecules. Assuming the hydrogen atoms in HCH as the electropositive partner interacting with the electronegative cyano group of the stationary phase, the retention behavior of the HCH isomers is well in agreement with expectations. The surface area formed by interacting hydrogen atoms undisturbed by chlorine increases from the α- via γ- to the β- and δ-isomers. Mononitro-PAHs were detected mainly in F13 and continued to elute in F14, where also dinitro-PAHs occurred. Keto- and ketohydroxy-PAHs with parental molecular weight up to 228 (anthraquinone, 9*H*-fluoren-9-one, benz[*a*]anthracene-7,12-dione) were observed in F14, whereas N-PACs were spread over F14 to F17. In F15, benzo[*a*]pyrene-7,8-dione and 3-hydroxybenzo[*a*]pyrene were quantified. Eleven other dione derivatives and nine other monohydroxy derivatives of PAHs with molecular weight 252 were tentatively identified in the same fraction.

Industrial chemicals with potentially estrogenic and/or other endocrine-disrupting activities (such as dialkyl phthalates, alkylphenols, several musk compounds, and bisphenol A) as well as a number of compounds with more than one polar functional group (aminoanthraquinone, *N*,*N*-diethylcarbanilide, prometryn) were found in the semipolar to polar fractions F14 to F17. Furthermore, saturated and unsaturated aliphatic compounds containing polar functional groups such as ketones, acids, amides, aldehydes, alcohols, and aromatic compounds with aliphatic side chains

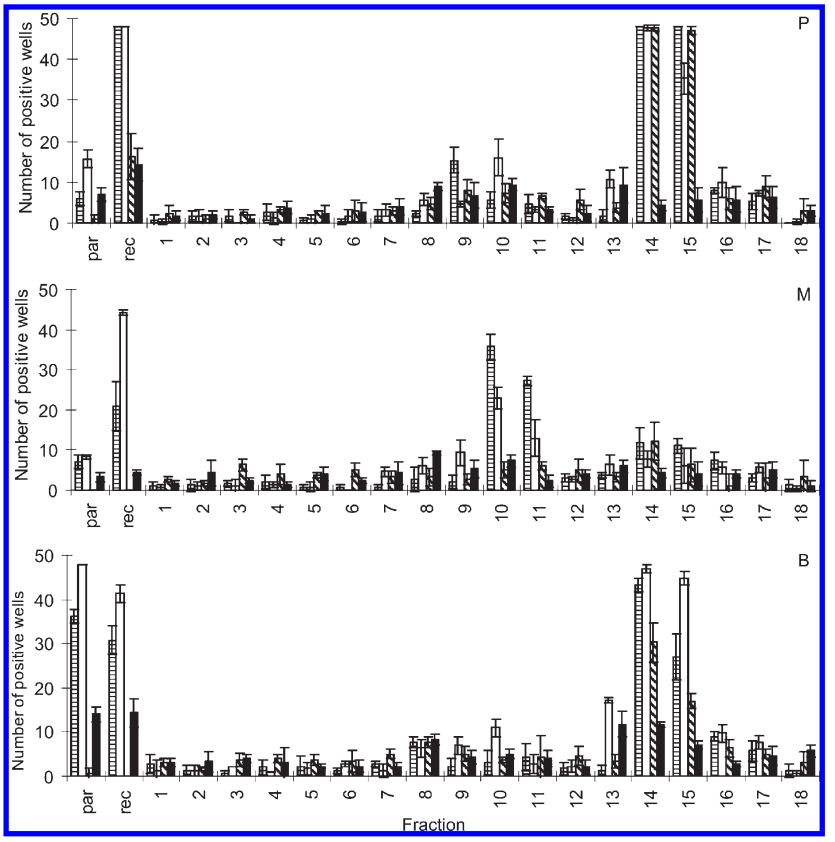

Figure 1. Mutagenic responses of total (par), reconstituted (rec), and fractionated (1−18) Přelouč (P), Most (M) and Bitterfeld (B) sediment extracts determined with tester strain TA98 without (horizontally shaded) and with (white) metabolic activation, and with TA100 without (diagonally shaded) and with (black) metabolic activation by S9. Concentration was 0.2 g sediment equivalents mL^{-1}. The maximum number of revertants is 48. 160 × 163 mm (300 × 300 dpi).

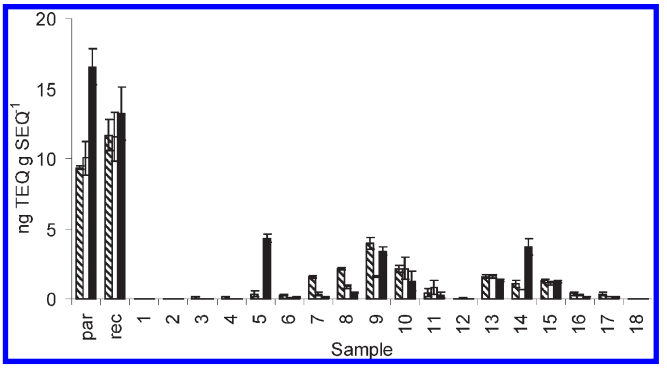

Figure 2. Aryl hydrocarbon receptor-mediated activities of parent (par), reconstituted (rec), and fractionated (1−18) Přelouč (shaded), Most (white) and Bitterfeld (black) sediment extracts expressed as ng toxicity equivalents (TEQ) per g sediment equivalents (SEQ)-1. 157 × 84 mm (600 × 600 dpi).

(dichlorobenzophenone, *N*-phenyl-2-naphthylamine, triphenyl phosphate, diphenyl sulfide) occurred in the semipolar to polar F13 to F18.

In Vitro Biological Screening. *Mutagenicity.* Mutagenicity of all fractions as well as the parent and reconstituted extracts was examined in a liquid microplate version of the Ames assay using tester strains TA98 and TA100 with and without metabolic activation at a test concentration of 0.2 g SEQ mL^{-1} (Figure 1). The comparison of responses of reconstituted samples containing equal amounts of each fraction of the corresponding sample with those of the parent extracts allows the determinations of activity losses occurring during sample processing. Reconstituted extracts of Přelouč and Most were more mutagenic than the parent extracts (see also Figure S3, Supporting Information) possibly due to losses of suppressing compounds by irreversible binding to stationary phases.[12,13] More probably, precipitation during solvent exchange removed compounds masking mutagenic effects due to cytotoxicity, inhibition of biochemical processes in the cells, or in S9 enzyme activity, or by reducing bioavailability.[14−16] Significant mutagenicity of precipitates was excluded by testing in the Ames assay.

In general, equal or higher responses were observed for TA98 than for TA100. Both direct and indirect mutagenic effects were found primarily in fractions 9 to 11 and F13 to 17 with focus on fractions 10, 11, 14, and 15 (Figure S1, S2, Table S2, Supporting Information). Fractions 9 to 11 contained well-known mutagenic PAHs with four to six aromatic rings (Table S1). In addition, methylated PAHs which may be even more mutagenic than their parent compounds[17,18] are expected to contribute to mutagenicity. Because most PAHs are known to be mutagenic only after metabolic activation,[19] other compounds such as directly acting pentafused-PAHs[20,21] might be responsible for the observed direct mutagenic effect. Nitro- and dinitro-PAHs such as 1-nitropyrene and 1,3- and 1,8-dinitropyrene detected in F13 and F14 were found to be potent direct and indirect acting mutagens in *Salmonella typhimurium*.[22,23] Furthermore, methyl-parathion, aminoanthraquinone, and dibenzoacridines detected in F14, F15, and F17, respectively, have been reported to be

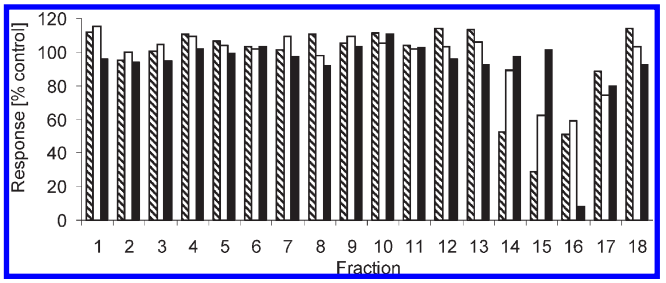

Figure 3. Acute inhibition of gap junction expressed as % of the control. Responses shown for fractions 1−18 of Přelouč (shaded), Most (white), and Bitterfeld (black) sediment extracts and a concentration of 0.1 mg sediment equivalents mL^{-1}. 157 × 79 mm (600 × 600 dpi).

mutagenic compounds in the Ames assay. No or only weak mutagenicity of certain oxy-PAHs (benz[*a*]anthracene-7,12-dione, phenanthrenol, benzofluorenones, anthrone) has been reported in the tester strain TA98.

AhR-Mediated Activity. The DR-CALUX assay was used to determine AhR-mediated activities. The assay showed a number of biologically active fractions with site-specific effect patterns (Figure 2; Figure S4, Supporting Information). Only minor differences between the parent and the reconstituted extracts were observed in the DR-CALUX assay, indicating that no significant losses of AhR-inducing compounds have occurred during the fractionation process. The activity of the parent extracts from Přelouč and Most were quite similar (about 10 ng TCDD-EQ g SEQ^{-1}) while the Bitterfeld sample exhibited about 17 ng TCDD-EQ g SEQ^{-1}). The Bitterfeld sediment exhibited the greatest effects in F5 that coelutes with PCDD/Fs, coplanar PCBs, and PCNs that are known to be potent AhR-inducers.[24,25] Bitterfeld is well-known for significant PCDD/F, PCB, and PCN levels contributing to AhR-mediated effects in this area.[26−29] Sediments from Přelouč and Most exhibited only minor AhR-mediated activities in F5, suggesting only low levels of persistent PCDD/Fs and coplanar PCBs.

The PAH-containing fractions 7 to 10 and 13 exhibited AhR-mediated activity in all sediments which is in agreement with expectations since PAHs are well-known AhR-inducing components.[30] Interestingly, Přelouč fraction 7 contained parent PAHs without potency to induce the AhR-mediated activity but particularly high concentrations of alkylated dibenzothiophenes or naphthothiophenes as well as large amounts of alkylated fluorenes, phenanthrenes, and anthracenes (Table S1), which may contribute to the effects. The highest AhR-mediated activities were found in fractions 9 and 10. Fraction F9 contained monomethylated benz[*a*]anthracenes and chrysenes as major AhR agonists. Additionally, the moderatly AhR-inducing compounds chrysene and benz[*a*]anthracene contribute to effects as well. In F10, parent and monomethylated benzo[*a*]pyrenes and benzofluoranthenes contribute to AhR activity. The prominent role of methylated PAHs for AhR-mediated effects of sediment extracts has been recently shown for other sediments.[31] Bitterfeld sediments also exhibited outstanding activity in the semipolar fraction F14 containing a large number of keto, hydroxy, amino, and nitro compounds. The most abundant oxygenated PAHs such as anthraquinone and benz[*a*]anthracene-7,12-dione as well as 7*H*-benz[*de*]anthracen-7-one and 3-hydroxybenzo[*a*]pyrene found in F14 and F15 of all sediments were reported to be only weak AhR-agonists in the DR-CALUX. Semipolar fractions F14 and

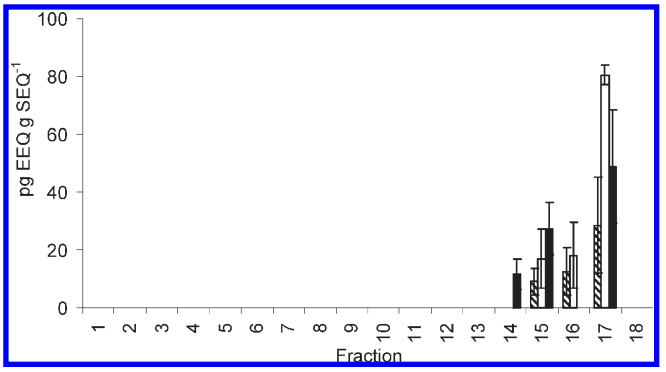

Figure 4. Estrogen receptor-mediated activity obtained for fractions 1−18 of the Přelouč (shaded), Most (white), and Bitterfeld (black) sediment extracts expressed as 17β-estradiol equivalents (EEQ) per g SEQ-1. 159 × 86 mm (600 × 600 dpi).

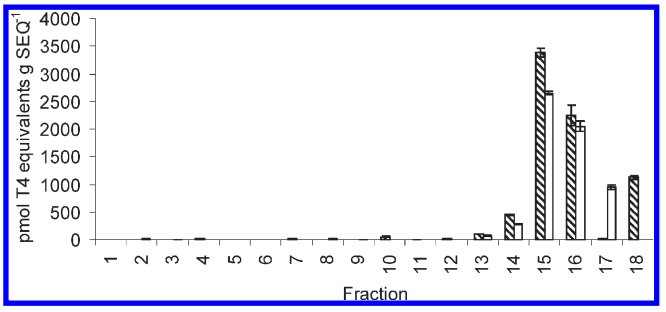

Figure 5. TTR binding potency expressed as pmol T4 equivalents per g sediment equivalent (SEQ) in fractions 1−18 of sediment extracts from Přelouč (shaded) and Most (white). 156 × 79 mm (600 × 600 dpi).

F15 also contributed to AhR-mediated effects in Přelouč and Most sediments.

Inhibition of GJIC. Potencies of the fractions to block GJIC were determined in scrape loading/dye transfer assay using rat liver epithelial WB-F344 cells. At the test concentration of 0.1 g SEQ mL^{-1}, significant inhibitions of GJIC were found exclusively after exposure to semipolar and polar fractions F14 to F17 (Figure 3) with Bitterfeld F16 and Přelouč F15 showing the strongest inhibitory potencies (down to 8% and 29%, respectively, see also Figure S5, Supporting Information). Although individual parent and methylated PAHs including low-molecular-weight compounds and noncoplanar PCBs are known inhibitors[3,32] of gap junctional communication, these compounds did not significantly contribute to GJIC inhibition. Since only very limited information on the GJIC inhibition potency of polar chemicals is available, no conclusions on the cause of the effect may be drawn.

Estrogenicity. The estrogenicity of sediment fractions was determined in the ER-CALUX assay (Figure 4, Figure S6, Supporting Information). Whereas nonpolar fractions exhibited no significant ER-mediated activity at a concentration up to 10 mg mL^{-1} SEQ, semipolar and polar fractions F14 to F17 showed significant ER-activation and ER-dependent gene transcription. The highest responses up to 81 pg 17β-estradiol (EEQ) g SEQ^{-1} was exhibited by Most F17; other fractions (Bitterfeld F15 and F17, Přelouč F17) showed responses between 27 pg EEQ g SEQ^{-1} and 49 pg EEQ g SEQ^{-1}. Known contributors were estrone in fractions 16 as well as 17β-estradiol in fractions 15 and 16, 17α-ethinylestradiol in Bitterfeld F17 and

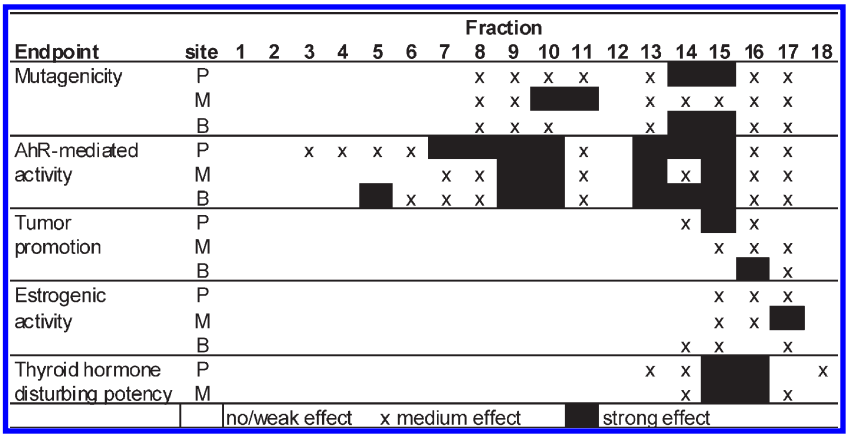

Figure 6. Toxicity pattern of 18 fractions obtained from the Přelouč (P), Most (M), and Bitterfeld (B) sediment extracts. 64 × 27 mm (600 × 600 dpi).

7*H*-benz[*de*]anthracen-7-one in Přelouč F15. Other industrial contaminants such as alkylphenols, anthraquinone, benz[*a*]anthracene-7,12-dione, bisphenol A, and musk compounds may only weakly contribute to the overall ER-mediated activity.

TTR-Binding Potency. Přelouč and Most fractions were tested for TTR binding activities in the TTR binding assay. Thyroid hormones are associated to transport proteins such as transthyretin (TTR) and are essential for proper development and differentiation of all cells. In vitro binding studies of several groups of environmental contaminants have shown that some of them are able to bind to TTR and compete with target hormones, with potencies comparable or even higher than the natural ligand, T4.[33−37] Thyroxine equivalents were derived from dose−response relationships at test concentrations of 0.6 g SEQ mL^{-1} to 60 g SEQ mL^{-1} (Figure 5). In this study, no or low activities were determined for the nonpolar fractions. Whereas somewhat higher activities were observed for Přelouč and Most F13 and F14, the majority of the TTR-binding activity was recovered in the polar fractions, particularly in F15 and F16 with responses up to 3385 pmol T4 equivalents g SEQ^{-1}. High potency was also found in Most F17. This strong effect was not observed for Přelouč F17 but was for Přelouč F18, which was a nonactive Most fraction. Only a few compounds listed in Table S1 were tested for their TTR-binding activity. Whereas biphenyl[34] penta- and hexachlorobiphenyl,[38] *o,p′*- and *p,p′*-DDE, DDD, and DDT,[39] and bisphenol A [34,40] as well as benzo[*a*]pyrene[40] were inactive, 4-nonylphenol[34,40] contained in fractions 14 and 15, respectively, showed TTR-binding activities. Since functional groups are needed to bind to the transthyretin active site,[41] many aromatic ketones and hydroxylated PAHs identified in F15 and F16 could be of interest as possible TTR-binding compounds. This needs to be confirmed by additional bioassay analyses.

Toxicity Patterns and Identification of Priority Fractions. An overall evaluation based on five in vitro toxicological end points relevant for mutagenicity, endocrine disruption, and reproductive effects (Figure 6) reveals similar toxicity patterns for different sediments despite different concentrations of individual components in these samples. The evaluation strongly highlights the significance of semipolar to polar fractions (F13 to F17) for hazards due to contaminated sediments. These fractions caused moderate to strong mutagenic, AhR-mediated, GJIC, estrogenic, and thyroid hormone-disrupting effects in all investigated samples. Sediment risk assessment is normally based on nonpolar compounds such as PAHs, PCBs, and PCDD/Fs. These compounds contribute to mutagenicity and AhR-mediated effects. However, it becomes obvious that this kind of assessment ignores a major fraction of hazardous compounds. This fraction becomes even more dominant if bioavailability is considered in prioritization and risk assessment.[42,43] Polar fractions of sediment contamination are much less defined than the nonpolar fractions and contain a multitude of compounds with diverse functional groups. Many of these compounds are not identified yet or lack data on their potential to cause adverse effects. Thus, these fractions should be given a major focus in sediment analysis and assessment in order to come up with more realistic figures of risks due to contaminated sediments.

■ ASSOCIATED CONTENT

Supporting Information. Additional data on sampling sites, applied bioassays, analytical procedures and results, recoveries of toxic effects, dose−response relationships, and cellular toxicity. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49 341 235 1531; fax: +49 341 235 2401; e-mail: werner.brack@ufz.de.

Present Addresses

#Current address: Joint Research Centre, European Commission, Institute for Environment and Sustainability, Via Enrico Fermi 2749, 21027 Ispra, Italy.

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